IFN-β Expression Is Directly Activated in Human Neutrophils Transfected with Plasmid DNA and Is Further Increased via TLR-4-Mediated Signaling.

IFNβ expression is directly activated in human neutrophils transfected with plasmid DNA and is further increased via TLR-4-mediated signalling

Running title: *IFNβ mRNA regulation in transfected neutrophils*

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Abstract

Upon LPS binding, TLR4 activates a MyD88-dependent pathway leading to the transcriptional activation of proinflammatory genes, as well as a MyD88-independent/TRIF-dependent pathway, responsible for the transcriptional induction of IFNβ. Previous findings have delineated that human neutrophils are unable to induce the transcription of IFNβ in response to TLR4 stimulation. Since neutrophils do not express PKCε, a molecule recently reported as essential for initiating the MyD88-independent/TRIF-dependent pathway, we optimized an electroporation method to transfect PKCε into neutrophils with very high efficiency. By doing so, a significant IFNβ mRNA expression was induced, in the absence of LPS-stimulation, not only in PKCε-overexpressing neutrophils but also in cells transfected with a series of empty DNA plasmids: LPS, however, further upregulated the IFNβ transcript levels in plasmid-transfected neutrophils, regardless of PKCε overexpression. Phosphoimmunoblotting studies, as well as chromatin immunoprecipitation assays targeting the IFNβ promoter, revealed that IFNβ mRNA induction occurred through the cooperative action of IRF3, activated by transfected DNA, and NF-κB, activated by LPS. Additional immunoblotting and coimmunoprecipitation studies not only revealed that neutrophils constitutively express various cytosolic DNA sensors, including IFI16, LRRFIP1 and DDX41, but also identified IFI16 as the intracellular receptor recognizing transfected DNA. Consistently, infection of neutrophils with intracellular pathogens such as *Bartonella henselae*, *Listeria monocytogenes*, *Legionella pneumophila* or adenovirus type 5 promoted a marked induction of IFNβ mRNA expression. Taken together, data raise questions on the role of PKCε in driving the MyD88-independent/TRIF-dependent response, while they indicate that human neutrophils are able to recognize and respond to microbial cytosolic DNA.

Introduction

The innate immune system, to which polymorphonuclear neutrophils (PMN) belong, is highly specialized in its capacity to recognize foreign pathogens, due to the expression of evolutionary conserved families of receptor proteins, the "pattern recognition receptors" (PRR) (1). The latter include, among others, the Toll like receptors (TLRs)(2), which all are constitutively expressed and functional in human neutrophils, with the exception of TLR3 (3-5). For instance, TLR4, which is the specific receptor for endotoxin (6), potently triggers TNFα, CXCL8, CCL3, CCL4, CCL19, CCL20, IL-1ra and IL-12p40 mRNA expression and production in neutrophils stimulated with lipopolysaccharide (LPS)(7). However, unlike human monocytes, endotoxin-activated neutrophils neither express type I IFN (IFNβ and/or IFNα), nor IFN-dependent genes (IRG), such as CXCL10 or CXCL9 (8). This has been recently attributed to the inability of neutrophils to mobilize the so-called "myeloid differentiation factor-88 (MyD88)-independent/TIR domain-containing adapter inducing IFNβ (TRIF)-dependent" pathway (8). In fact, it has emerged from studies mainly performed in macrophages or dendritic cells of gene-targeted mice, that LPS triggers two classes of genes *via* TLR4 (1). One class is defined as "MyD88-dependent" because it is not induced in MyD88-/- mice, and mostly includes proinflammatory mediators such as TNFα, IL-1, IL-12p40, and CXCL8 (1). In this pathway, MyD88 and TIRAP/MAL (TIR domain containing adapter protein/myeloid differentiation factor-88 adapter-like protein) mediate a rapid and early activation of the transcription factor NF-κB, which is essential for transcriptional induction of the above-mentioned proinflammatory genes (1). The other class of genes is defined as "MyD88-independent", because it relies on a more delayed activation of both NF-κB and IRF (IFN-regulatory factor)-3 transcription factors in MyD88-/- mice, that ultimately lead to the expression of IFNβ and, subsequently, to an IFNβ-dependent STAT1 activation (1). They include, for instance, a number of antiviral genes, Th1-activating chemokines such as MIG (monokine induced by IFNγ)/ CXCL9, IP-10 (IFN-inducible protein-10)/CXCL10

and I-TAC (IFN-inducible T-cell alpha chemoattractant)/ CXCL11 and anticancer molecules such as TRAIL (TNF-related apoptosis-inducing ligand) (9). Along the "MyD88-independent" pathway, TRAM (TIR domain-containing adapter-inducing IFNβ-related adapter molecule) and TRIF (TIR domain-containing adapter inducing IFNβ) adaptor proteins both transduce the activation of redundant protein kinases, namely TBK1 (TRAF family associated NF-κB binding kinase [TANK]-binding kinase-1) and IKK (IκB kinase, IKK)-ε, which phosphorylate IRF-3 on Ser/Thr residues (10). As a result, IRF-3 dimerizes, translocates to the nucleus, associates with other coactivators and ultimately contributes to activate IFNβ gene transcription (11). Interestingly enough, the "MyD88-independent/TRIF-dependent" cascade seems to be initiated by a rapid activation of PKCε in response to LPS which, according to recent findings, would lead to phosphorylation of TRAM on serine residues (12). Such a post-translational modification would then cause TRAM disappearance from the membrane, in concomitance with the activation of a downstream signaling cascade that would finally lead to the activation of the TBK1-IRF3 axis and ultimately the transcriptional induction of IFNβ (12).

In this work, given the absence of PKCε in human neutrophils (13-16), we verified whether the lack of PKCε expression could represent the precise defect that prevents the activation of the LPS-triggered MyD88-independent/TRIF-dependent pathway. For this purpose, we developed a transfection procedure to overexpress PKCε in human neutrophils and ultimately test whether we could rescue the MyD88-independent/TRIF-dependent pathway. While our experiments disprove a role of PKCε in driving the MyD88-independent/TRIF-dependent response, they helped us to subsequently demonstrate that, similarly to all cell types tested to date, also human neutrophils constitutively express various cytosolic DNA sensors. Consequently, we could show that neutrophils can promptly express IFNβ mRNA upon recognition of transfected DNA, or upon infection with intracellular pathogens such as *B. henselae*, *L. monocytogenes*, *L. pneumophila* or adenovirus type 5.

Materials and Methods

Antibodies – PKCε (sc-214), phospho-PKCε (Ser 729) (sc-12355), PKCβII (sc-210), IRF-3 (sc-9082), NF-κB p65 (sc-372), NF-κB p50 (sc-7178), IκB-α (sc-371), IFI16(sc-8023), DDX41 (sc-166225) and LRRFIP1 (sc-135917) antibodies (Abs) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). TBK1 (no. 3013), phospho-IRF3 (Ser 396) (no. 4947), phospho-NF-κB p65 (Ser536) (no. 3031) and phospho-p44/42 MAPK (no. 9106) Abs were from Cell Signaling (Beverly, MA, USA), while anti-β-tubulin (T5293) and anti-actin (A5060) Abs were from Sigma (Saint Louis, MO, USA). TBK1 monoclonal mAbs (IMG-139A) and rabbit polyclonal STING Abs (IMG-6422A) were from Imgenex (San Diego, CA, USA), while rabbit polyclonal ISG15 Abs were kindly provided by Dr. Arthur L. Haas (Louisiana State University Health Sciences Center, New Orleans, LA, USA).

Cell purification and culture – Highly purified granulocytes (neutrophils > 96.5 %, eosinophils < 3 %, n=30) and Percoll-purified monocytes (n= 15) were isolated and prepared under endotoxin-free conditions from buffy coats of healthy donors, as previously described (17). Ficoll-Paque isolated neutrophils were further enriched by positively removing any eventual contaminating cells, to reach > 99.7 % purity (high purity neutrophils), at least once for each type of experiments presented in this paper (18). Immediately after purification, neutrophils were either subjected to the transfection procedure (see below) or suspended in RPMI-1640 medium supplemented with 10 % low endotoxin FBS (< 0.5 EU/ml, BioWhittaker, Verviers, Belgium), treated with or without stimuli [including 100 ng/ml ultrapure *Escherichia coli* LPS (0111:B4, from Alexis), 10 μM R848 (InvivoGen), 50 μg/ml poly(I:C) (InvivoGen), 10 ng/ml phorbol-myristate acetate (PMA) (Sigma) or 100 U/ml IFNγ (R&D Systems, Minneapolis, MN, USA)], and then plated either in 6/24-well tissue culture plates (Nunc, Roskilde, Denmark), or in polystyrene flasks (Orange, Trasadingen, Switzerland) to be cultured at 37° C, 5% CO_2 atmosphere. After the desired incubation

period, cells were collected, spun at 300 × *g* for 5 min and cell pellets either extracted for total RNA or lysed for protein analysis as described below. HEK293T cells (German Collection of Microorganism and Cell Cultures, Braunschweig, Germany) were cultured in 24-well plates using DMEM medium (Lonza) supplemented with 10 % FBS and transfected with 2 μl of lipofectamine 2000 (Invitrogen) complexed with 0.8 μg poly(dA:dT) (Sigma) or pEGFP (Clontech). All reagents used were of the highest available grade and were dissolved in pyrogen-free water for clinical use.

Transfection of neutrophils – Neutrophils were transfected with different types of plasmids, including pmaxGFP (Lonza), pEGFP and pEGFP-PKCε [kindly provided by Professor P. Parker (Cancer Research UK, London Research Institute)] previously purified using the EndoFree Plasmid Maxi Kit (QIAGEN), as well as with *E. Coli* DNA (#D4889, RNA- and contaminant-free, according to the Sigma datasheet, as well as to our own checking), poly(dA:dT) and poly(I:C). Transfection was performed using the human monocyte nucleofector kit (Lonza) and the Amaxa nucleofector II device (Lonza) according to the reagent instructions, yet with minor modifications. After isolation, 5 x 10^6 neutrophils were resuspended in 100 μl of complete nucleofector solution containing 2.5-15 μg DNA (or equal volumes of PBS, for mock-transfection) and then transferred to a nucleoporation cuvette. Electroporation was performed using the Y001 program present in the nucleofector II device. Cells were then recovered and let stand for 5 min in 2 ml of human monocyte nucleofector medium supplemented with 2 mM glutamine and 10 % FBS. Thereafter, neutrophils were washed once with PBS to remove broken cells and then subjected to flow cytometry analysis using a FACScan flow cytometer (Becton-Dickinson, Franklin Lakes, NJ) to determine GFP expression. In selected experiments, transfected-neutrophils were preincubated for 30 min with different inhibitors, including BAY 117082, pyrrolidine

dithiocarbamate (PDTC), sc-514 (Calbiochem, San Diego, CA) or 10 μM MG132 (Sigma), prior to the subsequent stimulation with LPS.

Reverse transcription quantitative real-time PCR (RT-qPCR) – RNA isolation and reverse transcription were accomplished as previously described (19). RT-qPCR was performed using SYBR® Premix Ex Taq (Takara) and gene-specific primers (purchased from Invitrogen, Carlsbad, CA, USA) available in the public database RTPrimerDB (http://medgen.ugent.be/rtprimerdb/index.php) under the following entry codes: IFNβ (3542), TNFα (3551), CXCL8/IL-8 (3553), CXCL10/IP-10 (3537), G1P2/ISG15 (3547), IFIT1 (3540), and GAPDH (3539). Data were calculated with Q-Gene software (www.BioTechniques.com) and are expressed as mean normalized expression (MNE) units after GAPDH normalization.

Apoptosis assessment. Apoptosis of transfected neutrophils was determined as previously described (5), by the propidium iodide (PI) staining procedure, according to the "quick method" described by Riccardi *et al*. (20). Data were analysed by FLOWJO software (Tree Star Inc., Ashland, OR).

Immunoprecipitations and immunoblots – Whole cell extracts were prepared either using the RNeasy mini kit (Qiagen) as described (21), for immunoblots, or according to the chromatin immunoprecipitation (ChIP) assay procedure (22), for IFI16, LRRFIP1 and DDX41 immunoprecipitations. Cytoplasmic extracts for PKCε and TBK1 immunoprecipitations were prepared by the nitrogen cavitation procedure and processed exactly as previously described (23). Blotted proteins were detected and quantified using the Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE, USA) (24).

ChIP Assays – ChIP experiments were performed as described elsewhere (22), with minor modifications. For immunoprecipitation of transcription factors, nuclear extracts from 10^7 sonicated neutrophils were incubated with 7 μg of anti-IRF3 or 5 μg of anti-NF-κB p65/p50 Abs, while for cytoplasmic DNA sensor/plasmid DNA co-immunoprecipitations, whole cell extracts, prepared from 5 x 10^6 pEGFP-transfected neutrophils, were immunoprecipated using 5 μg of αDDX41, αIFI16 and αLRRFIP1 Abs. In all ChIP experiments, protein recruitment to the prolactin (PRL) promoter (a gene that is completely silent in our cell types) was used as negative control. The co-immunoprecipitated material was then subjected to qPCR analysis using the following specific primers (purchased from Invitrogen): IFNβ promoter forward: TCCCAGGAACTCAATGAAGG; IFNβ promoter reverse: GTGTCGCAATGGAGTGTTGT; CXCL8 promoter forward: CTTAGTGGGGTTGAAAGTGAC; CXCL8 promoter reverse: AAGAAATAGTCACTCACCCAAG; pEGFP-backbone forward: ACGGCATCAAGGTGAACTTC; pEGFP-backbone reverse: GCTTCTCGTTGGGGTCTTTG; pEGFP-GFP forward: TGCCATAGCCTCAGGTTACTC; pEGFP-GFP reverse: GACGCTCAGTGGAACGAAAAC. PRL promoter forward: AGGGAAACGAATGCCTGATT; PRL promoter reverse: GCAGGAAACACACTTCACCA. pEGFP-backbone and pEGFP-GFP primers of pEGFP (Accession Number: U55763) amplify, respectively, the 3854-3999 bp and the 1091-1259 bp regions.

Infection of neutrophils – 2 x 10^6 high purity neutrophils were infected with 10 MOI *L. monocytogenes*, *L. pneumophila* or *B. henselae*, and then cultured in 24-well tissue culture plates for up to 6 h prior to RNA extraction and RT-qPCR analysis. At 1 h post-infection, 50 μg/ml gentamicin was added to limit the growth of extracellular bacteria. In other experiments, neutrophils were infected with 1000 MOI adenovirus type 5 [E1-deleted, E3-

defective, encoding for GFP (AdV-GFP, a kind gift by Prof. B.M. Foxwell (Imperial College, London, UK)]. Bacterial strains were all grown to mid-logarithmic phase as it follows: wild type *L. monocytogenes* (WT, ATCC 19115) were grown in brain-heart infusion medium (Difco, Detroit, MI); *L. pneumophila* [serogroup 1 NCTC 12821, kindly provided by Prof. M.C. Zotti (University of Torino, Italy)] were grown in N-(2-acetamido)-2-aminoethanesulfonic acid buffered yeast extract broth supplemented with 0.4 mg/ml L-cysteine and 0.135 mg/ml ferric nitrate (Sigma); *B. henselae* Houston-1 (from ATCC 49882) were grown in Schneider's medium supplemented with 10 % FCS and 2 mM glutamine, at 37° C with 5 % CO_2 (25). Prior to infection, bacteria were pelleted and washed twice with PBS.

Statistical analysis – Data are expressed as means ± SE. Statistical evaluation was performed using student *t* test or 1-way ANOVA followed by Tukey's *post hoc* test. Values of $P < 0.05$ were considered statistically significant.

Results

Overexpression of PKCε in human neutrophils by electroporation – Immunoblot and immunoprecipitation analyses of either cytoplasmic cavitates (26) (Supplemental Fig. 1A, *top panel*), or whole cell lysates (not shown), confirmed (13-16) that neutrophils do not express PKCε, unlike autologous monocytes. Moreover, PKCε protein expression in both neutrophils and monocytes was found to be, respectively, neither inducible nor upregulated, by a 24 h-treatment with either LPS or IFNγ (Supplemental Fig. 1B). Therefore, to clarify whether the absence of PKCε might be responsible for the inability of human neutrophils to mobilize the "MyD88-independent/TRIF-dependent" pathway, we attempted to overexpress PKCε in these cells. A series of preliminary experiments were, however, necessary to develop a reliable transfection protocol, since neutrophils are notoriously extremely difficult to manipulate for exogenous gene expression, due to their inability to proliferate and to survive in culture for long periods. We were ultimately successful by modifying a recently reported nucleofection protocol (27), with which the authors were able to transfect both GFP and $p47^{phox}$-GFP proteins into neutrophils. By our procedure, we could transfect a pmaxGFP plasmid into neutrophils with a greater efficiency than the reported 5 % (27) (see the fluorescence microscopy image displayed in the *left* panel of Supplemental Fig 2A). Accordingly, flow cytometry analysis revealed that the percentage of GFP-positive neutrophils augmented in correlation with increasing amounts of transfected pmaxGFP plasmid (Supplemental Fig. 2B). For example, more than 50 % of GFP-positive neutrophils were measured by nucleofecting 15 μg pmaxGFP plasmid (Supplemental Fig. 2B). Importantly, such remarkable levels of GFP-positive neutrophils were obtained as early as 4 h post-nucleofection and remained stable for up to 20 h of culture (Supplemental Fig. 2C). However, since neutrophils became mostly apoptotic after 20 h (nearly 80 % were PI-positive) (Supplemental Fig. 2D), whereas they were substantially alive up to 7-8 h post-

transfection (75-80 % of PI-negative cells) (Supplemental Fig. 2D), all subsequent experiments were performed using neutrophils that were first transfected for 4 h and then stimulated with LPS for no more than 3 h.

The validity of our experimental procedure was confirmed by transfecting neutrophils with a plasmid encoding a GFP-tagged full-length PKCε (pEGFP-PKCε), which produced a *de novo* expression of remarkable quantities of exogenous PKCε-GFP (Fig. 1A-B). Indeed, pEGFP-PKCε-transfected neutrophils displayed specific PKCε–immunoreactive signals, unlike the cells transfected with the corresponding empty plasmid (pEGFP) (Fig. 1A). Again, expression levels of exogenous PKCε correlated with the amounts of transfected plasmid (Fig. 1B): for instance, the levels of exogenous PKCε-GFP expressed in 2 x 10^6 neutrophils transfected with 15 μg PKCε-GFP plasmid were comparable to the levels of endogenous PKCε present in 1 x 10^6 monocytes (Fig. 1A-B). Importantly, PKCε-GFP overexpressed in neutrophils underwent serine phosphorylation in response to PMA stimulation, as revealed by the use of specific anti-phospho-PKCε Abs (Fig. 1C). Similar data could be extrapolated by using anti-PKCε Abs which detect a doublet (28), the slower migrating band likely corresponding to phosphorylated PKCε – as clearly evidenced in monocytes (Fig. 1D). Importantly, transfected neutrophils displayed an unaltered capacity to respond to external agonists, as proven by the findings that PMA triggered ERK phosphorylation in electroporated neutrophils at levels substantially similar to those observed in non-transfected cells (Fig. 1C). Taken together, our data demonstrate that it is possible to successfully transfect PKCε-GFP in neutrophils and that exogenous PKCε-GFP can be rapidly phosphorylated by PMA.

LPS acquires the capacity to upregulate IFNβ mRNA expression in plasmid DNA-transfected neutrophils – To assess whether transfected PKCε could restore the "MyD88-

independent/TRIF-dependent" pathway, neutrophils were incubated 4 h post PKCε-GFP- or pEGFP-plasmid transfection and then stimulated with 100 ng/ml ultrapure LPS for additional 90 min (see the *upper panel* Figure 2A), to investigate the state of IFNβ and CXL10 mRNA expression by RT-qPCR. In line with our previous study (8), no IFNβ or CXL10 mRNA induction occurred in non-transfected neutrophils stimulated with LPS, as opposed to the induction of two classical MyD88-dependent genes, namely TNFα and CXCL8 (Fig. 2A, *lower* panels). By contrast, both IFNβ and CXCL10 (but not TNFα or CXCL8) gene expression was significantly increased by LPS in PKCε-GFP-overexpressing neutrophils (Fig. 2A, *lower panels*), as if the "MyD88-independent/TRIF-dependent" pathway was rescued by the introduction of an exogenous PKCε. However, this presumed rescue also occurred in neutrophils transfected with a pEGFP plasmid, with increased amounts of IFNβ and CXCL10 transcripts in response to LPS at levels comparable to those measured in PKCε-GFP-overexpressing neutrophils (Fig. 2A, *center panels*). Unexpectedly, neutrophils transfected with either PKCε-GFP or pEGFP plasmids were found to express elevated amounts of IFNβ, CXCL10, TNFα and CXCL8 transcripts even in the absence of any LPS stimulation (Fig. 2A), thus indicating that the simple introduction of DNA into neutrophils strongly promotes their gene activation. Accordingly, data depicted in Fig. 2B clearly demonstrate that, upon transfection with pEGFP plasmid (or PKCε-GFP plasmid, or *E.coli* DNA, data not shown), a time-dependent increase of IFNβ and CXCL10 mRNA is already detectable in neutrophils after 45 min, that augments thereafter up to 7 h. Also neutrophil electroporation with poly(dA:dT), a chemically synthesized DNA commonly used to study the immunological response to B-DNA (29), was found to directly trigger IFNβ and CXCL10 mRNA expression (Fig. 2C, *lower panels*). The latter experiments not only demonstrate that the ability of neutrophils to recognize foreign DNA is not restricted to plasmid DNA, but also prove that the stimulating agent is genuine DNA and not a contaminating product derived

from the plasmid isolation procedure, such as for instance bacterial RNA. Moreover, a further stimulation with LPS of poly(dA:dT)-electroporated neutrophils again resulted in an upregulation in the levels of IFNβ and CXCL10 transcripts relative to non-transfected neutrophils (Fig. 2C, *lower panels*). Such an LPS-mediated effect was also observed in neutrophils transfected with *E. coli* DNA (Fig. 2C, *lower panels*), but, interestingly, not with poly(I:C) (Fig. 2C, *lower panels*), a synthetic analog of viral dsRNA which we previously showed to very strongly upregulate IFNβ mRNA expression when electroporated into human neutrophils (5). Altogether, our data demonstrate that the mere DNA/plasmid DNA transfection of neutrophils markedly activates the expression of IFNβ and CXCL10 mRNA. Our data also demonstrate that LPS further upregulates the expression of IFNβ and CXCL10 mRNA in DNA/plasmid DNA-transfected neutrophils, yet in a manner that appears independent from the overexpression of exogenous PKCε-GFP.

Identification of IRF3 as the critical factor for the transcription of IFNβ mRNA in plasmid DNA-transfected neutrophils – Subsequent experiments were aimed at elucidating the molecular bases responsible for the induction of IFNβ mRNA expression in neutrophils transfected with plasmid DNA, as well as for its further up-regulation by LPS. For such a purpose, we analyzed the activation status of IRF3, which represents a crucial intermediate for the transcriptional induction of IFNβ (11). By using the protocol depicted in the scheme of Figure 3A (*upper panel),* we found that the mere pEGFP-transfection of neutrophils triggered a direct *ser396* phosphorylation of IRF3 (Fig. 3A, *center panel*), that was relatively weak after 2 h but became very strong after 5 h (for a quantitative densitometric analysis see *lower panel* of Fig. 3A, n=3). Notably, similar results were obtained with the PKCε-GFP plasmid (data not shown), while a 1 h-stimulation of DNA-transfected neutrophils with LPS did not augment the levels of *ser396* phosphorylated IRF3 (Fig. 3A). Consistent with these

findings were the results from IRF3 ChIP assays, which revealed a strong recruitment of IRF3 to the IFNβ promoter in plasmid-transfected neutrophils (Fig. 3B, *lower panel*), and that such recruitment is not further increased by the subsequent LPS-stimulation (Fig. 3B, *lower panel*). The specificity of the IRF3 ChIP assay was proved by the fact that the PRL promoter was not amplified under the same experimental conditions (Fig. 3B, *lower panel*), and by the fact that matched control antibodies did not coprecipitate any IFNβ or PRL promoter (not shown). Altogether, data demonstrate that while the transfection of neutrophils with plasmid DNA directly activates the phosphorylation of IRF3 and its binding to the IFNβ promoter, LPS remains unable to modify the activation status of IRF3 even if it upregulates the accumulation of IFNβ transcripts. It follows that the upregulatory effect of LPS at the level of IFNβ gene expression in plasmid-transfected neutrophils does not occur *via* the "MyD88-independent/TRIF-dependent" cascade.

Identification of the transcription factors promoting the expression of IFNβ mRNA in plasmid DNA-transfected neutrophils upon LPS-stimulation: role of NF-κB – Since NF-κB is a transcription factor cooperating with IRF3 in promoting IFNβ transcription (30), we subsequently analyzed its activation status in plasmid-transfected neutrophils treated with or without LPS for 45 min (Fig. 4, *panels* A and B). By doing so, we observed considerable levels of phosphorylated NF-κBp65 (Fig. 4A) and diminished amounts of IκBα (Fig. 4B) in neutrophils transfected with DNA plasmids for 4 h and 45 min. Moreover, the addition of LPS to 4 h-plasmid transfected neutrophils resulted in a further increase of the levels of NF-κB p65 phosphorylation (Fig. 4A) and IκBα degradation (Fig. 4B) after 45 min (for a quantitative densitometric analysis see *lower panels* of Fig. 4A and 4B, n=3). Importantly, ChIP assays performed according to the scheme depicted in Fig. 4C, using antibodies towards NF-κBp65 (Fig. 4D) and NF-κBp50 (Fig. 4E), highlighted a crucial role of NF-κB in driving

the effect of LPS on IFNβ mRNA expression in plasmid-transfected neutrophils. In fact, both p50 and p65 NF-κB subunits were strongly recruited to the IFNβ promoter upon LPS-stimulation of plasmid-transfected neutrophils (Fig. 4D and 4E, *left panels*). On the other hand, no recruitment of either NF-κBp50 or NF-κBp65 to the IFNβ promoter was observed in non-transfected neutrophils treated with LPS (Fig. 4D and 4E, *left panels*), consistent with the inability of LPS to induce IFNβ gene expression under the latter conditions (8). Under the latter conditions, however, both NF-κBp65 and NF-κBp50 were find to strongly bind to the CXCL8 promoter (Fig. 4D and 4E, *right panels*). In plasmid-transfected neutrophils, notably, the recruitment of NF-κBp65 and NF-κBp50 to the CXCL8 promoter, although negligible after 60 min of LPS-stimulation (Fig. 4D and 4E, *right panels*), was at least three-fold higher after 30 min (data not shown), in line with the enhanced CXCL8 mRNA expression observed in parallel (Figure 2A).

The role of activated NF-κB as crucial mediator of the additional upregulation of IFNβ mRNA expression by LPS in plasmid-transfected neutrophils was additionally supported by the effect of four chemical NF-κB blockers, namely BAY 117082, an inhibitor of IκBα phosphorylation and degradation (31), PDTC, an inhibitor of NF-κB activation (32), sc-514, a selective IKK-2 inhibitor (33) (Fig. 5), and MG132, a proteasomal inhibitor (34) (not shown). In fact, while they had no effect on the induction of IFNβ mRNA directly triggered by the simple plasmid transfection, all of them significantly reduced the upregulatory effect of LPS on IFNβ mRNA expression (Fig. 5). All in all, our data suggest that the increased expression of IFNβ mRNA observed in response to LPS in plasmid transfected neutrophils is likely the consequence of the LPS-triggered recruitment of p65 and p50 NF-κB subunits to the IFNβ promoter, which, for unknown reasons, does not take place in not-transfected neutrophils.

Intracellular recognition of plasmid DNA by human neutrophils is dependent on IFI16 (interferon-inducible protein 16) – Finally, we aimed at defining how human neutrophils recognize transfected plasmid DNA. Initially, we investigated the role of TLR9, since this molecule is constitutively expressed and responsive in human neutrophils (4, 35). TLR9 recognizes unmethylated CG dinucleotides (CpG) present in the extracellular space within the endosomal compartment (36) and consequently drugs able to block endosome acidification (37), such as chloroquine and bafylomicin A1, inhibit its ability to activate NF-κB and to induce cytokines (37-38). Experiments in which neutrophils were pretreated with the two inhibitors for 30 min prior to plasmid DNA electroporation (for 4 h), revealed that both chloroquine and bafylomicin A1 do not prevent the induction of IFNβ or TNFα gene expression (Supplemental Fig. 3A, *left and center panels*). By contrast, both inhibitors markedly suppressed the induction of TNFα mRNA expression in R848-stimulated neutrophils (Supplemental Fig. 3A, *right panel*), therefore proving that they were appropriately functioning. Then, we tested the role of RNA polymerase III, which functions as an indirect intracellular DNA sensor (39-40). In fact, RNA polymerase III recognizes AT-rich DNA and consequently synthesizes uncapped 5′ triphosphate–bearing RNA which, in turn, serves as an agonist for retinoic acid-inducible gene (RIG-I) (39-40), that is constitutively expressed by human neutrophils (Fig. 6A) (5). Once again, a specific RNA polymerase III inhibitor-based approach, by using ML-60218, (39-40), did not suppress the induction of IFNβ mRNA exerted by plasmid DNA- or poly(dA:dT)-electroporation (Supplemental Fig. 3B,). By contrast, ML-60218 dose-dependently reduced the induction of IFNβ mRNA in 293T cells transfected with poly(dA:dT) (Supplemental Fig. 3C), as expected (39-40). We could thus conclude that neither TLR9, nor RNA polymerase III, function as the intracellular receptor(s) recognizing plasmid DNA in electroporated neutrophils. On the other hand, we could confirm that neutrophils express IFI16 (41), one of the newly identified intracellular receptor for foreign DNA (42). Accordingly, immunoblotting experiments

revealed that whole neutrophil and monocyte lysates contain IFI16, as a cluster of proteins of 85-95 kDa (Fig. 6A), similarly to what originally described in the nuclear extracts of IFNγ-treated HL-60 cells (41). Under the same conditions, we could also detect, in both neutrophils and monocytes, two more recently discovered DNA sensors, namely leucine-rich repeat (in Flightless I) interacting protein-1 (LRRFIP1), which promotes IFNβ gene expression through β-catenin engagement and not *via* IRF3 activation (43), and DDX41, a member of the DEXDc family of helicases (44) (Fig. 6A). Furthermore, we found that neutrophils also express stimulator of IFN genes (STING) (Fig. 6A), a transmembrane protein located in the endoplasmic reticulum that is crucial for DNA-mediated signalling (45-48).

To attempt identifying the specific DNA sensor(s) binding transfected DNA in neutrophils, we used the strategy that allowed Zhang *et al*. (44) to identify the intracellular binding protein recognizing *L. monocytogenes* DNA. Precisely, we immunoprecipitated IFI16, LRRFIP1 and DDX41 after DNA transfection (Fig. 6B), and then analyzed, by real-time qPCR, which of these receptors plasmid DNA was stably bound to (Fig. 6C). By doing so, we found that IFI16 functions as the intracellular DNA sensor that, in transfected neutrophils, is predominantly involved in recognizing plasmid DNA (Fig. 6C).

Induction of IFNβ mRNA expression by infection of neutrophils with intracellular pathogens – In a final series of experiments, we tested a number of intracellular pathogens, including *B. henselae*, *L. monocytogenes*, *L. pneumophila* and adenovirus type 5, that, according to the literature (49) are known to activate IFNβ gene expression *via* cytosolic sensing of their DNA. As shown in Figure 7A, all intracellular bacteria under examination triggered a remarkable IFNβ and CXCL10 mRNA expression in neutrophils infected for 6, but not 3, h. Similarly, all bacteria upregulated the expression of TNFα (Fig. 7A) and CXCL8 mRNA (data not shown), indicating they are able to activate a wide spectrum of neutrophil genes upon their recognition. A time-dependent induction IFNβ and CXCL10

mRNA expression was also observed in neutrophils infected with adenovirus type 5 (Fig. 7B). The latter phenomenon was genuine as testified by the amplification of transcripts encoding GFP, which are virus-specific (Fig. 7B).

Discussion

A peculiar feature that differentiates human neutrophils from monocytes in terms of LPS responsiveness is that neutrophils are unable to mobilize the MyD88-independent/TRIF-dependent cascade upon TLR4 engagement (8). In this study, in light of the identification of PKCε as a crucial molecule for initiating the MyD88-independent/TRIF-dependent" pathway (12), and having confirmed that PKCε results undetectable in human neutrophils (13-16), we attempted to overexpress PKCε with the aim to restore the "MyD88-independent/TRIF-dependent" pathway in human neutrophils. By optimizing a previously described electroporation method (27) to obtain more than 50 % GFP-positive cells, we were able to successfully transfect PKCε in neutrophils. However, despite of the very high amounts of exogenous PKCε protein expressed by transfected neutrophils, the results of our subsequent experiments did not permit us to conclude that overexpressed PKCε is instrumental in rescuing the "MyD88-independent/TRIF-dependent" pathway. In fact, while LPS did acquire the capacity to upregulate the expression of IFNβ and CXCL10 mRNA (as well as ISG15 and IFIT-1 mRNA, N.T. and M.A.C., unpublished observations) in PKCε-transfected neutrophils, a similar phenomenon was also observed in neutrophils transfected with *E. coli* DNA, poly(dA:dT), or a variety of empty plasmids (but not in mock-transfected cells). Moreover, the fact that LPS did not modify the state of IRF3 activation/phosphorylation in either plasmid- or PKCε–transfected neutrophils, further demonstrates that the MyD88-independent/TRIF-dependent cascade was not activated in DNA-nucleofected neutrophils. Assuming that overexpressed PKCε was fully functional in neutrophils (in this regard, we show that PKCε underwent serine phosphorylation upon cell treatment with PMA), a further implication of our results is that PKCε may not be as critical for the activation of the MyD88-independent/TRIF-dependent cascade as previously proposed (12). Consistent with such a notion, the role of PKCε in driving the MyD88-independent/TRIF-dependent response has

been recently questioned also by Parker *et al.* (50), who demonstrated that PKCε associates with MyD88 and that PKCε phosphorylation is important for the NF-κB activation *via* TLR2, which, unlike TLR4, does not utilize the TRIF or TRAM adaptors (51).

Whatever the case may be, herein we also show that the mere transfection of neutrophils with plasmid DNA (regardless of the presence of a PKCε coding region within the vector) directly activates IRF3, promotes its recruitment to the IFNβ promoter and, in turn, strongly induces the expression of IFNβ and other type I IFN-dependent genes (such as CXCL10, ISG15 and IFIT-1). Together with the findings described above, our data imply that, in plasmid-transfected neutrophils, LPS becomes able to further upregulate the expression of IFNβ mRNA without directly targeting IRF3. ChIP assays not only confirmed this hypothesis, but also established the presumed molecular mechanisms underlying the induction of IFNβ mRNA, either by plasmid transfection, or by LPS in plasmid-transfected neutrophils. Accordingly, we found that: i) plasmid DNA transfection promotes a time-dependent recruitment of IRF3 to the IFNβ promoter; ii) the addition of LPS to plasmid transfected neutrophils does not increase such IRF3 recruitment; iii) while cytosolic DNA markedly activates NF-κB in the cytoplasm, it is unable to promote NF-κB recruitment at the IFNβ promoter; iv) LPS promotes the binding of NF-κB to the IFNβ promoter in plasmid-transfected neutrophils but not in non-transfected cells; v) NF-κB inhibitors significantly suppress the upregulatory effect of LPS on IFNβ mRNA expression in transfected neutrophils. Based on these findings, we propose that the capacity of LPS to increase the expression of IFNβ mRNA in plasmid transfected neutrophils relies on its ability to activate NF-κB which, under conditions in which IRF3 is already present on the IFNβ promoter (i.e., after plasmid transfection), becomes readily recruited (see the scheme depicted in Supplemental Figure 4). In fact, given the closed proximity of the IRF3 binding site to the NF-κB locus within the IFNβ promoter, it is possible that the binding of NF-κB occurs only

when IRF3 is already bound. In this regard, it has been already demonstrated that the binding of IRF3 to the IFNβ promoter produces a nucleosome shift which functions as a prerequisite for the binding of NF-κB and AP1 to the IFNβ promoter (52). In other words, the nucleosome remodeling exerted by IRF3 might unmask the NF-κB binding sites present in the IFNβ promoter, in this way allowing NF-κB binding and consequent increased IFNβ transcription. All in all, our data are in accordance with the view that, even if not essential unlike IRF3 (11), NF-κB plays an important role in modulating the degree of IFNβ promoter activation (11, 53). This is also in line with other experimental systems in which the induction of IFNβ results from a cooperative action of two different signals (54-55). Similarly, the present data are consistent with previous findings demonstrating that human neutrophils often necessitate to be stimulated by two concurrent but different stimulatory pathways to optimally express a given gene: for instance IFNγ plus LPS for CXCL10 (56) and IL-12 (57) expression, or IL-10 plus LPS for IL-1ra (22) expression. Curiously, LPS was unable to further upregulate the levels of IFNβ and CXCL10 transcripts in poly(I:C)-transfected neutrophils. Although we did not specifically explore which are, at molecular levels, the reasons for such a LPS-inability, some speculations can be made. First, because we have shown that poly(I:C)-transfection itself represents one of the most potent stimulatory condition for gene expression in neutrophils (5), it is plausible that IFNβ and CXCL10 mRNA levels are already induced at the potential maximum levels, and are therefore not further increasable. Second, and in agreement with the former effects, poly(I:C)-transfection activates NF-κB so powerfully (5) that LPS might result unable to further activate it. However, the fact that LPS maintains the capacity to promote lactoferrin release (58) in poly(I:C)-transfected neutrophils (N.T. and M.A.C., unpublished observations) indicates that, under the latter experimental conditions, TLR4 is expressed and functional.

This study also demonstrates that human neutrophils can promptly respond to transfected DNA, whether of bacterial or synthetic origin. In this context, our observations complement a series of previous studies describing the capacity of neutrophils to respond to exogenous DNA. Neutrophils, in fact, can readily respond to CpG oligodeoxynucleotides (ODN) in terms of CXCL8 production and inhibition of apoptosis since they express their cognate receptor, TLR9 (4). Other findings have demonstrated that extracellular bacterial DNA can trigger, through an unidentified receptor, another pathway functioning in a TLR9- and CpG-independent but MyD88-dependent manner (59). Our new observations additionally suggest that human neutrophils possess intracellular sensor system(s) that allow(s) the recognition of foreign and potentially dangerous DNA, and consequently the induction of a distinct and potent immune response (60). Among the intracellular sensors that recognize exogenous DNA, that to date include TLR9 (36), RIG-I through RNA polymerase III (39-40), absent in melanoma 2 (AIM2) (61-64), LRRFIP1 (43), DNA-dependent activator of IRFs (DAI) (65), IFI16 (42) and DDX41 (44). Herein, we provide evidence that human neutrophils constitutively express, other than RIG-I (5), also DDX41, LRRFIP1 and IFI16, in the latter case confirming previous findings (41). We also show that neutrophils constitutively express STING, a transmembrane protein that is essential for the signaling necessary for the production of IFNβ, *via* IRF3 activation, mediated by various cytosolic DNA sensors (45, 66). In this context, we did not investigate the role of DAI, the first identified cytosolic DNA sensor (65), since wild type and DAI-deficient cells produce comparable amounts of type I IFN in response to cytosolic DNA (67) or poly(dA:dT) (68). We also did not focus on AIM2, a member of the PYHIN (pyrin and HIN200 domain-containing) protein family, since, based on the literature, this receptor is not essential for type I IFN induction by transfected DNA, while it is crucial for inflammasome activation and interleukin 1β (IL-1β) secretion (61-64). Moreover, we excluded the involvement of both TLR9 and RNA polymerase III by taking the advantage of using specific inhibitors. However, by performing co-immunoprecipitation

studies of DDX41, LRRFIP1 and IFI16 and, presumably bound, plasmid DNA, we were able to identify IFI16 as the critical intracellular sensor for plasmid DNA in human neutrophils. The latter findings are perfectly consistent with the biological features of IFI16 that, differently from TLR9, DAI and RNA pol III, does not recognize specific DNA structure (42), and that, through the activation of NF-κB and IRF3, is known to induce a strong production of proinflammatory cytokines and type I IFN in response to transfection with DNA motifs (42). The presence of RIG-I, DDX41 and LRRFIP1 in neutrophils provides, in any case, solid molecular bases explaining our additional findings on the capacity of *L. monocytogenes*, *L. pneumophila* and adenovirus type 5 to induce the expression of IFNβ and other ISG mRNA in infected neutrophils, since those pathogens require, for such a function, DDX41 plus LRRFIP1, RIG-I, and DDX41 plus RIG, respectively. On the other hand, we show that neutrophils express IFNβ mRNA also following infection with *B. henselae*, a gram-negative facultative intracellular microorganism that can invade many cells (69-70). Among others, *Bartonella* utilizes several virulence factors for its interaction with host cells, including the VirB/D4 type IV secretion systems (T4SSs), which has been recently shown to be able of mediating plasmid DNA and protein transfer into eukaryotic host cells (71). Since VirB/VirD4 T4SS is related to the dot/Icm T4SS used by *L. pneumophila*, which is essential to trigger IFNβ expression (72), likely via the RNA polIII/RIG cascade (40), it is therefore tempting to speculate that *B. henselae* might induce IFNβ mRNA through mechanisms similar to those utilized by *L. pneumophila*.

Whatever the case is, further studies are necessary to meticulously decipher which neutrophil sensor(s) is/are responsible for the recognition of foreign DNA from different sources, and in which cell compartment they locate to do so. Nonetheless, the findings of this paper shed new light on our understanding of the mechanisms by which human neutrophils recognize and respond to intracellular pathogens and, in turn, activate innate immune responses.

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Footnotes

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Figure legends

FIGURE 1. Transgenic expression of PKCε-GFP in human neutrophils. (A) Neutrophils were electroporated with 15 μg pEGFP-PKCε, 15 μg pmaxGFP or PBS (i.e., mock electroporated) as described in M&M. After 4 h, neutrophil and monocyte whole cell extracts were prepared and, together with extracts from HEK 293T cells, transfected with pEGFP-PKCε or not, were electrophoresed (50 μg neutrophils, 30 μg monocytes, 10 μg for HEK 293T) for immunoblotting studies using antibodies specific for PKCε and PKCβII. Panels depict a representative experiment out of three independent ones. **(B)** Neutrophils were electroporated with 5 or 15 μg of pEGFP-PKCε, or with 15 μg of pEGFP. After 4 h, whole cell extracts were prepared and then electrophoresed for PKCε and actin immunoblotting. Panels depict a representative experiment (n=2). **(C)** Neutrophils were electroporated with 15 μg of pEGFP-PKCε for 4 h, treated with 5 ng/ml PMA and then lysed after additional 20 min. Whole cell extracts were prepared, electrophoresed and immunoblotted using antibodies specific for phospho-PKCε, total PKCε, phospho p42/p44 and actin. **(D)** Whole cell extracts prepared from monocytes stimulated for 20 min with 5 ng/ml PMA were processed as for **(C)** and used as positive control. For **(C, D)**, data show one experiment representative of two ones.

FIGURE 2. Modulation of IFNβ mRNA expression in plasmid transfected-neutrophils incubated with or without LPS. Neutrophils were electroporated with 15 μg pEGFP-PKCε, 15 μg pEGFP (empty vector) **(A, B)**, 15 μg of poly(dA:dT), 15 μg *E.coli* DNA, 2 μg of poly(I:C) or PBS (mock electroporated) (**C)**, and then incubated either for 4 h **(A, C)** before the addition of 100 ng/ml LPS, or for the times indicated only **(B)**. Total RNA was extracted after further 90 (**A)** or 180 min (**C)** post LPS-stimulation, or as indicated **(B)**, and then

analyzed for IFNβ, CXCL10, TNFα, CXCL8 and GAPDH mRNA expression by RT-qPCR. In the *upper parts* of panels (**A)** and (**C)** are reported the schemes illustrating the experimental protocols utilized. Gene expression is depicted as MNE ± SE (n=6) after GAPDH normalization of triplicate reactions for each sample. Asterisks indicate a significant increase exerted by LPS. * $p<0.05$, *** $p< 0.001$. For **B** and **C**, depicted results are from one representative experiment of at least three independent ones.

FIGURE 3. Activation of IRF3 in plasmid-transfected neutrophils. (A) Neutrophils were electroporated with 15 μg pEGFP and cultured for up to 5 h, the last hour in the presence or the absence of 100 ng/ml LPS. Neutrophils were lysed at the 2 and 5 h-time point (as depicted in the *upper* scheme) and whole cell extracts then electrophoresed and immunoblotted using anti-phosphoIRF3 (Ser 396), anti-IRF3 and anti-actin Abs. The *center panel* shows a representative immunoblot (n=3), while the *lower panel*, displays the densitometric quantification of phospho-IRF3 levels (normalized by the total IRF3, n=3) by LI-COR Odyssey software, expressed as densitometric units (D.U.). Asterisks indicate a significant increase of phospho-IRF3 levels over not-transfected cells. * $p<0.05$, ** $p< 0.01$. **(B)** Neutrophils were electroporated with 15 μg pEGFP and cultured for 5 h, the last hour in the presence or the absence of 100 ng/ml LPS. Cells were then processed for ChIP analysis as described in M&M. Enrichment of IFNβ and PRL promoters in coprecipitated DNA was analyzed by qPCR using promoter-specific primers. Data from qPCR are expressed as percentages over input DNA (mean ± SE, n=3). Asterisks indicate a significant increase of IRF3 recruitment over not-transfected cells. * $p<0.05$.

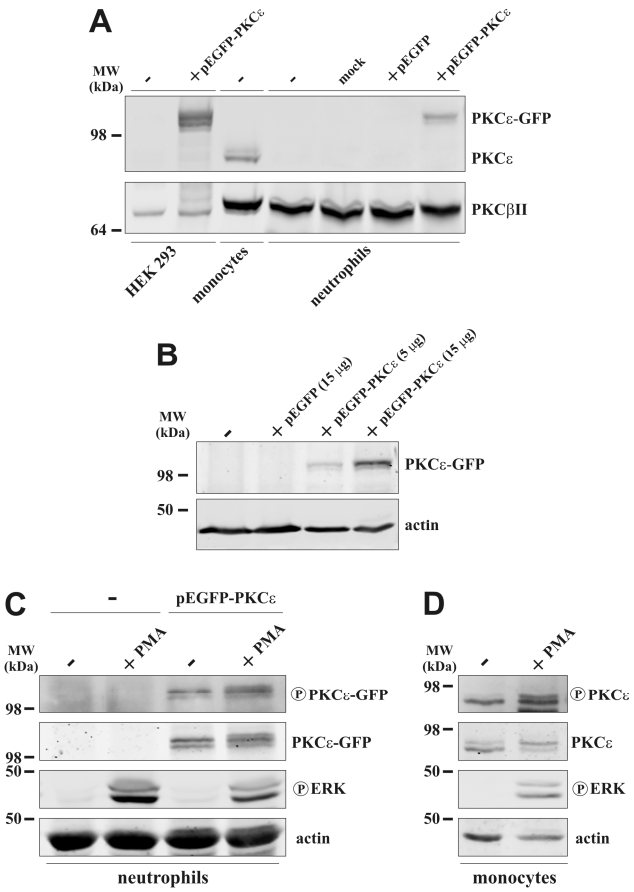
FIGURE 4. LPS-mediated NF-κB activation and binding to the IFNβ-promoter in plasmid-transfected neutrophils. (A, B) Neutrophils were electroporated with 15 μg

pEGFP and cultured for 4 h and 45 min, the last 45 min in the presence or the absence of 100 ng/ml LPS (as depicted in the *upper* schemes). Whole cell extracts were prepared, electrophoresed and immunoblotted using anti-phospho-NF-κBp65 (Ser536), anti-total NF-κBp65 **(A)**, anti-IκBα and anti-actin antibodies **(B)**. Shown is a representative immunoblot split into two parts (n=3). The *lower* graphs in **(A)** and **(B)** illustrate the densitometric quantification of the phospho-NF-κBp65 levels (normalized by the total NF-κBp65) **(A)**, and of the IκBα levels (normalized by the total actin) **(B)**, expressed as mean ± SE (n=3). Asterisks indicate significant differences among the various experimental conditions (* $p<0.05$, ** $p< 0.01$, *** $p< 0.001$), as indicated. For panels **(D)** and **(E)**, neutrophils were treated as depicted in the scheme of panel **(C)**, and processed for ChIP analysis using anti-NF-κBp65 **(D)**, or anti-NF-κBp50 **(E)** Abs. Enrichment in IFNβ, CXCL8 and PRL promoter in coprecipitated DNA was analyzed by qPCR using promoter-specific primers. Data from qPCR are expressed as percentages over input DNA (mean ± SE, n=3). Asterisks indicate a significant increase of NF-κB recruitment over control or not-transfected cells. ** $p<0.01$, *** $p< 0.001$.

FIGURE 5. Effect of NF-κB inhibitors on the upregulation of IFNβ mRNA expression triggered by LPS in plasmid-transfected neutrophils. Neutrophils were electroporated with 15 μg of pEGFP and, after 3.5 h of culture, incubated with 300 μM PDTC, 100 μM sc-514 or 5 μM BAY 117082. After 30 min, neutrophils were stimulated with 100 ng/ml LPS for further 90 min (as depicted in the *upper* scheme). Total RNA was then extracted and analyzed for IFNβ and GAPDH mRNA expression by RT-qPCR. IFNβ mRNA expression is depicted as MNE ± SE (n=3) after GAPDH normalization of triplicate reactions for each sample. Asterisks indicate a significant inhibition by the inhibitor. ** $p<0.01$, *** $p< 0.001$.

FIGURE 6. Neutrophil recognition of intracellular plasmid DNA is dependent on IFI16. **(A)** Whole neutrophil and monocyte extracts (60 μg) were electrophoresed and immunoblotted using antibodies specific for IFI16, LRRFIP1, DDX41 RIG-I, STING and tubulin. **(B)** Whole neutrophil lysates (500 μg), prepared using the ChIP assay buffer, were subjected to immunoprecipitation with IFI16, LRRFIP1, DDX41 or anti-mouse IgG1 (control) antibodies. For **(A)** and **(B)**, representative immunoblot experiments are shown (n=3). **(C)** Neutrophils were transfected with pEGFP, cultured for 3 and 5 h, and then disrupted by ChIP assay buffer. Whole cell lysates were then subjected to immunoprecipitation with IFI16, LRRFIP1, DDX41 or anti-mouse IgG1 control antibodies. Finally, co-immunoprecipitated DNA was purified and analyzed by qPCR using two different sets of primers, specific for pEGFP (pEGFP-backbone and pEGFP-GFP). Data from qPCR are expressed as percentages over input DNA. Results are from one experiment representative of four.

FIGURE 7. Induction of IFNβ mRNA expression in neutrophils infected by various intracellular pathogens. (A) Neutrophils were infected for 3 and 6 h with *B. henselae*, *L. monocytogenes*, *L. pneumophila* at MOI 10. Total RNA was then extracted and analyzed for IFNβ, CXCL10, TNFα and GAPDH mRNA expression by RT-qPCR. **(B)** Neutrophils were infected with adenovirus type 5 at MOI 1000. Total RNA was extracted after 1.5 and 5 h of incubation and analyzed for IFNβ, CXCL10, GFP and GAPDH mRNA expression by RT-qPCR. Gene expression in **A** and **B** is depicted as MNE after GAPDH normalization of triplicate reactions for each sample (MNE ± SE). Depicted results are from one experiment representative of at least three independent ones.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MW (kDa)
-
+pEGFP-PKCε
-
-
mock
+pEGFP
+pEGFP-PKCε
98
64
PKCε-GFP
PKCε
PKCβII
HEK 293
monocytes
neutrophils
B
MW (kDa)
-
+ pEGFP (15 µg)
+ pEGFP-PKCε (5 µg)
+ pEGFP-PKCε (15 µg)
98
50
PKCε-GFP
actin
C
-
pEGFP-PKCε
MW (kDa)
-
+ PMA
-
+ PMA
98
98
50
50
Ⓟ PKCε-GFP
PKCε-GFP
Ⓟ ERK
actin
neutrophils
D
MW (kDa)
-
+ PMA
98
98
50
50
Ⓟ PKCε
PKCε
Ⓟ ERK
actin
monocytes

Figure 1

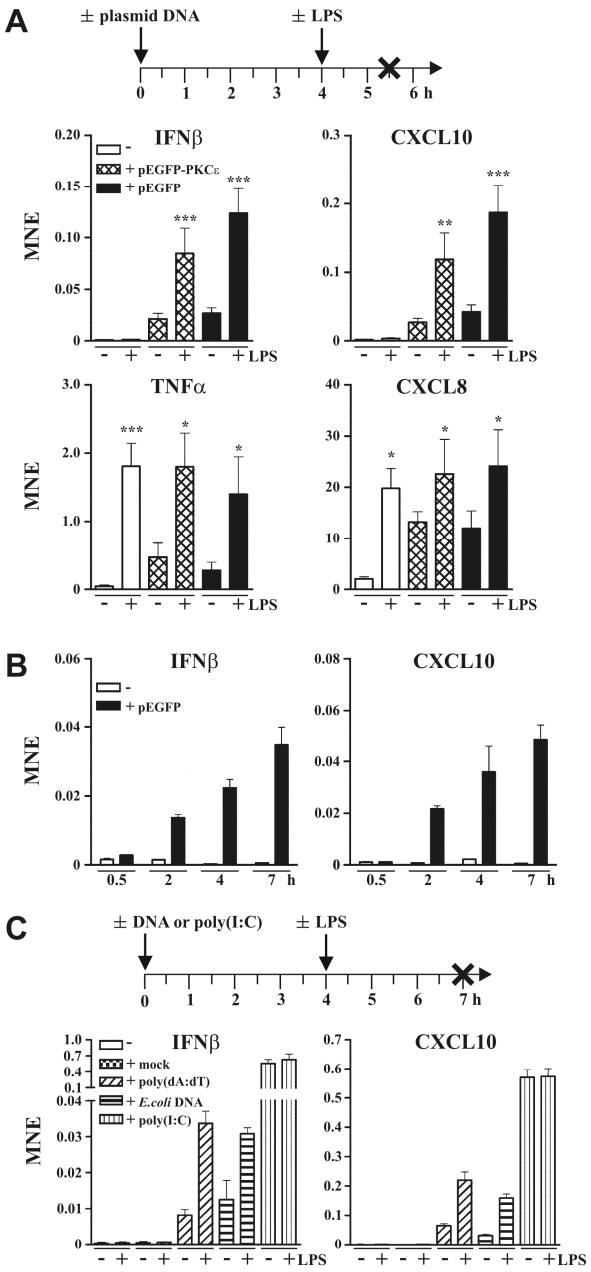

A
± plasmid DNA
± LPS
0 1 2 3 4 5 6 h
IFNβ
CXCL10
- pEGFP-PKCε
+ pEGFP
MNE
TNFα
CXCL8
LPS
B
IFNβ
CXCL10
- pEGFP
0.5 2 4 7 h
C
± DNA or poly(I:C)
± LPS
0 1 2 3 4 5 6 7 h
IFNβ
CXCL10
- mock
+ poly(dA:dT)
+ E.coli DNA
+ poly(I:C)
LPS

Figure 2

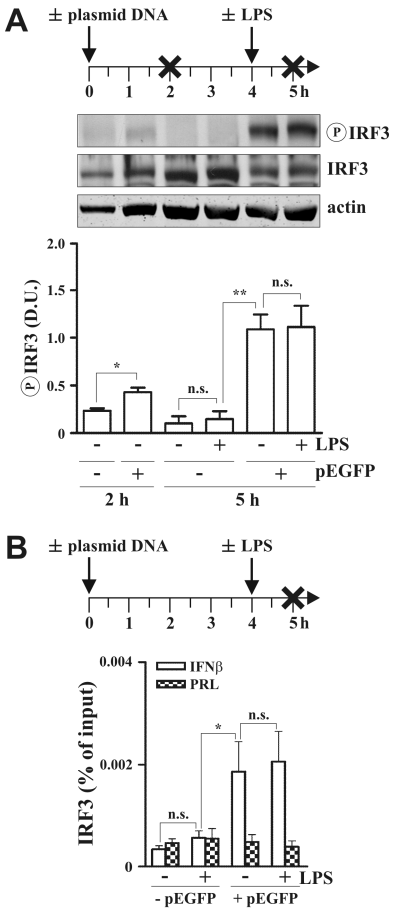

A
± plasmid DNA
± LPS
0 1 2 3 4 5h
Ⓟ IRF3
IRF3
actin
Ⓟ IRF3 (D.U.)
2.0
1.5
1.0
0.5
0
*
n.s.
**
n.s.
- - - + - + LPS
- + - + pEGFP
2 h
5 h
B
± plasmid DNA
± LPS
0 1 2 3 4 5h
IRF3 (% of input)
0.004
0.002
0
IFNβ
PRL
n.s.
*
n.s.
- + - + LPS
- pEGFP
+ pEGFP

Figure 3

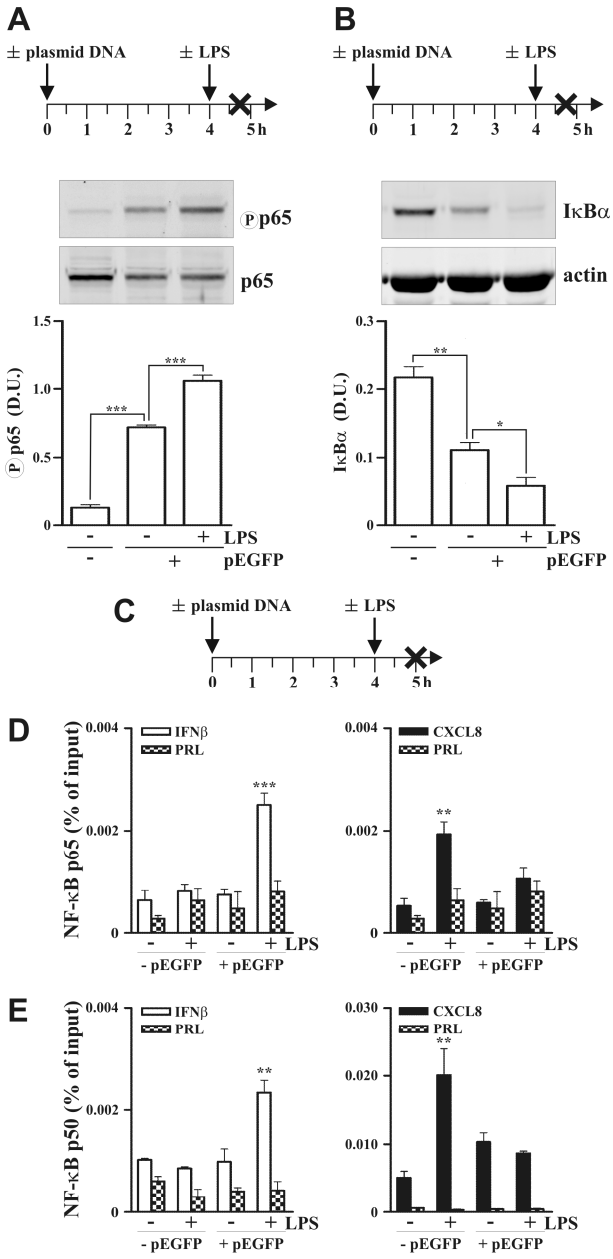

A
± plasmid DNA
± LPS
0 1 2 3 4 5h
P p65
p65
P p65 (D.U.)
LPS
pEGFP
B
± plasmid DNA
± LPS
0 1 2 3 4 5h
IκBα
actin
IκBα (D.U.)
LPS
pEGFP
C
± plasmid DNA
± LPS
0 1 2 3 4 5h
D
IFNβ
PRL
NF-κB p65 (% of input)
CXCL8
PRL
LPS
- pEGFP
+ pEGFP
E
IFNβ
PRL
NF-κB p50 (% of input)
CXCL8
PRL
LPS
- pEGFP
+ pEGFP

Figure 4

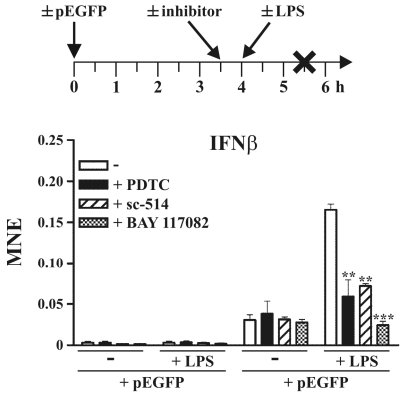


Figure 5

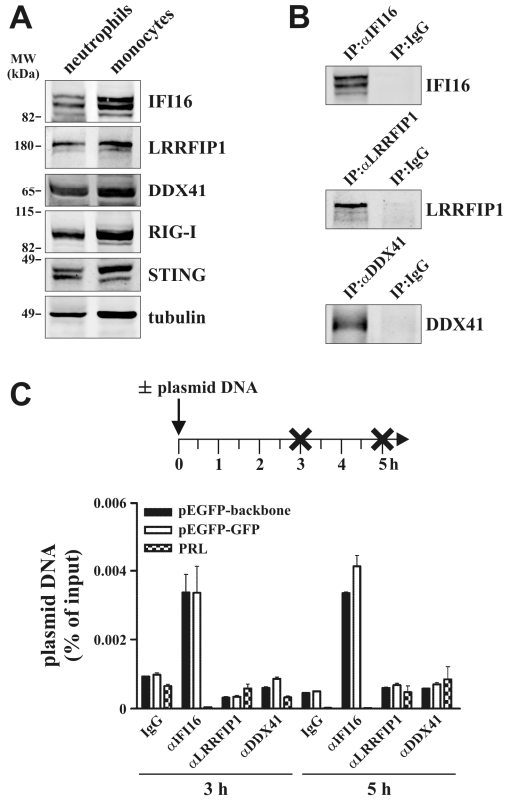


Figure 6

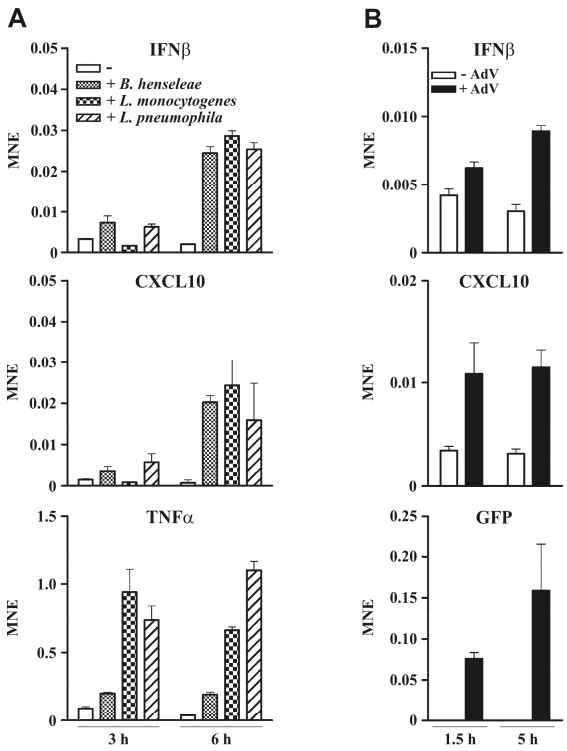
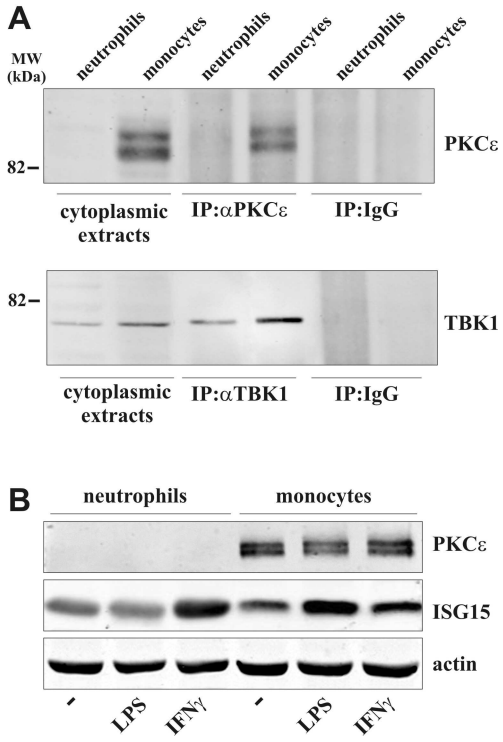

A
IFNβ
-
+ B. henseleae
+ L. monocytogenes
+ L. pneumophila
MNE
0.05
0.04
0.03
0.02
0.01
0
CXCL10
0.05
0.04
0.03
0.02
0.01
0
TNFα
1.5
1.0
0.5
0
3 h
6 h
B
IFNβ
- AdV
+ AdV
0.015
0.010
0.005
0
CXCL10
0.02
0.01
0
GFP
0.25
0.20
0.15
0.10
0.05
0
1.5 h
5 h

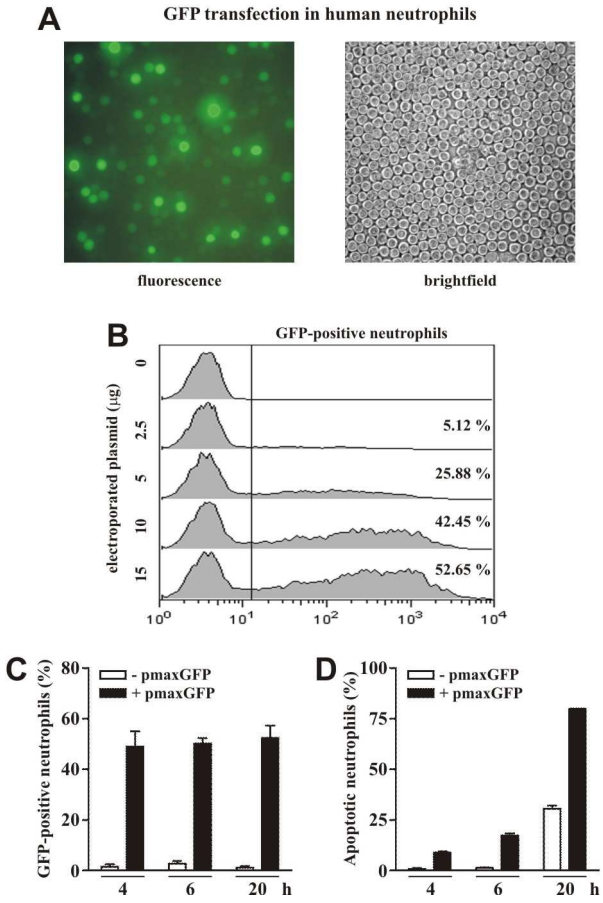
Figure 7

Supplemental Figure 1



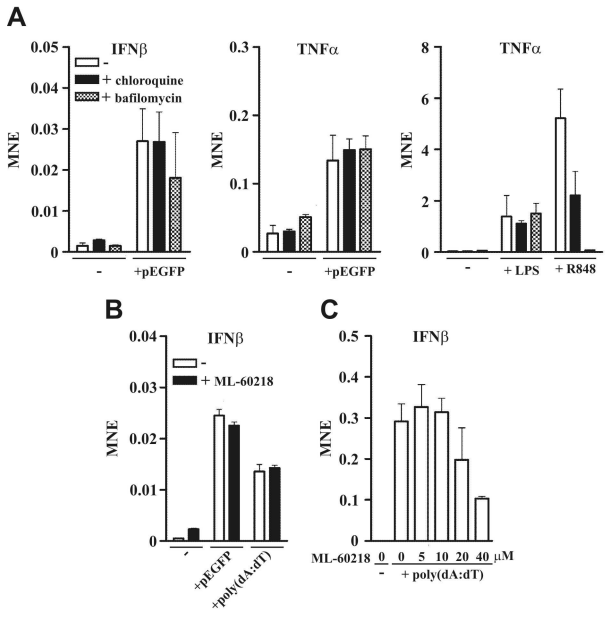
Supplemental Figure 1. PKCε protein is not detectable in human neutrophils. (A) Cytoplasmic extracts prepared by nitrogen cavitation (see M&M) from neutrophils and autologous monocytes were either electrophoresed (50 μg neutrophils, 30 μg monocytes) or immunoprecipitated (500 μg from neutrophils, 300 μg from monocytes) with specific anti-PKCε and TBK1. Samples were also immunoprecipitated with anti-rabbit IgG control antibodies. A representative immunoblot experiment out of three with similar results is shown. Panel **(A)** shows that PKCε is not detectable in either cytoplasmic neutrophil cavitates (*upper* panel). On the other hand, TBK1, which has a MW comparable to that of PKCε, is readily detected in neutrophil samples (*lower* panel), proving that our samples were correctly prepared. **(B)** Neutrophils and autologous monocytes were incubated with 100 ng/ml LPS or 100 U/ml IFNγ for 24 h. Whole cell extracts were then prepared, electrophoresed (50 μg neutrophils, 30 μg monocytes) and immunoblotted using antibodies specific for PKCε, ISG15 and actin. Panels display one experiment representative of three with similar results. Data demonstrate that the levels of ISG15, but not PKCε, protein are upregulated by IFNγ in both neutrophils and monocytes, as well as by LPS in monocytes only, in line with the fact that ISG15 is a "MyD88-independent/TRIF-dependent" gene.

Supplemental Figure 2



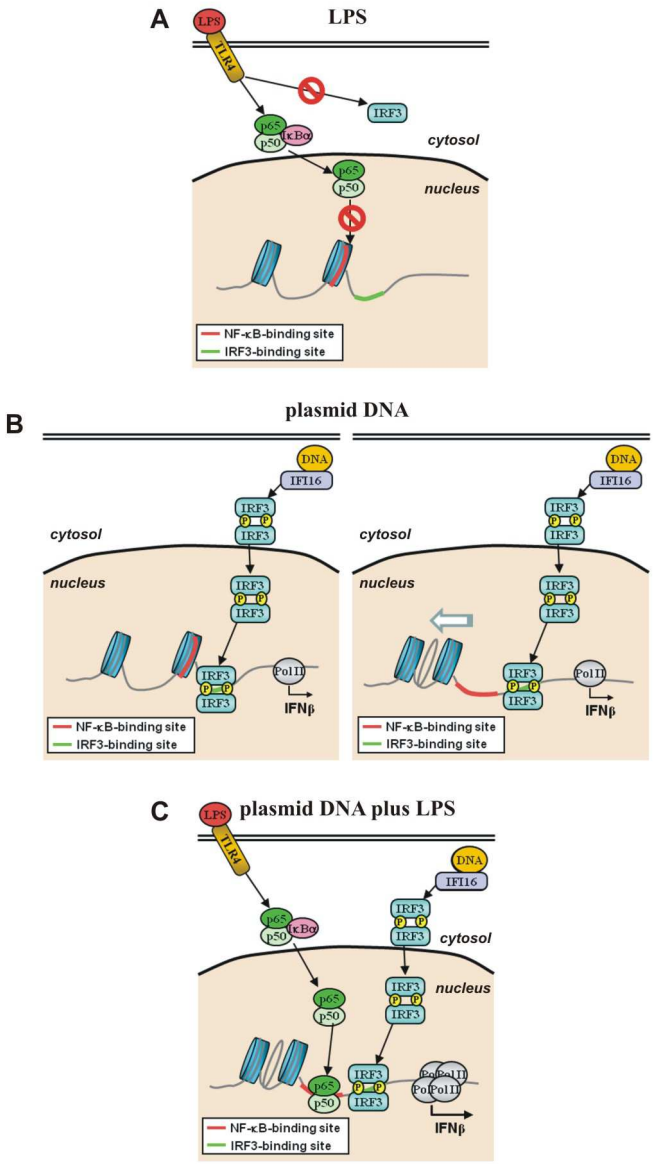
Supplemental Figure 2. Expression of transfected green fluorescent protein (GFP) in human neutrophils. (A) Human neutrophils were electroporated with a 15 μg GFP-encoding plasmid (pmaxGFP) as described in M&M and, after 6 h, cell images were captured by a fluorescence microscope, either in fluorescence (left panel), or in bright field (right panel), mode. **(B)** Neutrophils were electroporated with different amounts of pmaxGFP plasmid prior to measuring GFP expression levels after a 4 h-culture, by flow cytometry. Values within each plot indicate the percentage of GFP-positive neutrophils. A representative experiment out of three with similar results is shown. **(C)** Neutrophils were electroporated with 15 μg pmaxGFP plasmid and expression of GFP protein was detected after 4, 6 and 20 h by flow cytometry. Values (mean ± SE, n=3) indicate the percentage of GFP-positive neutrophils. **(D)** Neutrophils were treated as described for panel **C** to evaluate their apoptotic state after different incubation times, as measured by the propidium iodide staining method. Values are expressed as mean ± SE (n= 3).

Supplemental Figure 3



Supplemental Figure 3. Recognition of intracellular DNA by human neutrophils is independent from TLR9 or RNA polymerase III. (A) Neutrophils were pre-treated for 30 min with 5 μg/ml chloroquine or 25 nM bafylomicin A1 and then either electroporated with 15 μg of pEGFP (*left* and *center* panels**)**, or incubated with 100 ng/ml LPS or 10 μM R848 for 90 min (*right* panel**)**. After 5 h (*left* and *center* panels**)**, total RNA was extracted and analyzed for IFNβ (*left* panels**)**, TNFα (*right* and *center* panels**)** and GAPDH mRNA expression by RT-qPCR. **(B)** Neutrophils were pre-treated for 30 min with 30 μM ML-60218 and then electroporated with either 15 μg pEGFP or 15 μg poly(dA:dT). After 5 h, total RNA was extracted and analyzed for IFNβ and GAPDH mRNA expression by RT-qPCR. **(C)** HEK 293T cells were pre-treated for 2 h with different doses of ML-60218 and then transfected with 0.8 μg/ml poly(dA:dT) complexed with lipofectamine. After 24 h, total RNA was extracted and analyzed for IFNβ and β2-microglobulin (β2m) mRNA expression by RT-qPCR. For all panels**,** gene expression is depicted as MNE after GAPDH (**A-B**) or β2m (**C**) normalization of triplicate reactions for each sample. Results are from one experiment representative of at least three independent ones.

Supplemental Figure 4



Supplemental Figure 4. Intracellular plasmid DNA activates IRF3 and favours the recruitment of LPS-activated NF-κB to the IFNβ promoter in human neutrophils. (A) Upon stimulation of human neutrophils with LPS, p65/p50 NF-κB heterodimers, even if activated *via* the MyD88-dependent pathway, do not bind to the IFNβ promoter since their binding sites are likely masked. No IRF3 activation occurs in LPS-stimulated neutrophils since the TRIF-dependent pathway is not functional. **(B)** Upon transfection, plasmid DNA binds to IFI16 and activates a signal transduction cascade that leads to IRF3 phosphorylation, dimerization and nuclear translocation. IRF3, in turn, is recruited to its binding sites within the IFNβ promoter to induce IFNβ transcription. Concomitantly, IRF3 presumably unmasks NF-κB recognition sequences within the same IFNβ promoter *via* activation of unknown, local chromatin remodelling events. **(C)** As a result, p65/p50 NF-κB heterodimers activated by LPS in DNA-transfected neutrophils become now able to bind the unmasked NF-κB

recognition sequences and, cooperating with IRF3, promote the recruitment of more RNA polymerase II and consequently increase IFNβ mRNA transcription.